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# A Liquid Assay for Screening Fungal Virulence Factors & *kgb-1*; *fer-1* Double Mutant Construction

Muxun Zhao

*Worcester Polytechnic Institute*

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**A Liquid Assay for Screening Fungal Virulence Factors**  
**&**  
***kgb-1;fer-1* Double Mutant Construction**

A Major Qualifying Project

Submitted to the faculty

Of

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

In Biology and Biotechnology

Biochemistry

By

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Muxun Zhao

Submitted and Approved on  
December, 2008

By

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Samuel M. Politz Ph.D, Project Advisor

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Reeta Prusty Rao Ph.D, Project Advisor

## Abstract

It is difficult to develop drugs against fungal infections due to the fact that both fungi and their hosts are eukaryotic. By using a host-pathogen system and an assay in high-throughput screens of genetic knockouts, fungal virulence factors as well as host immunity modulators can be identified and studied. We are developing a liquid culture assay for studying the effects of co-culturing *Caenorhabditis elegans* with *Saccharomyces cerevisiae* mutants. We tested several different conditions including different liquid media, *E.coli* to Yeast mass ratios, and *C. elegans* mutant strains. From the results of all the experiments, we concluded that there are other factors that can cause the death of the *C. elegans* in the assay, such as matricide, which are not *S. cerevisiae*-related. To exclude matricide in the experiment, a new *C. elegans* strain with *kgb-1*; *fer-1* double mutant genotype was constructed by crossing.

## Acknowledgements

We would like to thank Professors Sam Politz and Reeta Prusty Rao for their help, direction and insight on this project. We would also like to thank Charu Jain for her help in the lab, and Meijiang Yun for her help with Sigma Stat and information on her unpublished *kgb-1* survival data.

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## Introduction

In this project, we are developing a liquid culture assay for studying the effects of co-culturing *Caenorhabditis elegans* with *Saccharomyces cerevisiae* mutants. This host-pathogen model system allows us to do a high-throughput screen of the entire yeast genome. We expect to identify the fungal virulence factors involved in fungal infection, as well as host immunity modulators for innate immunity study.

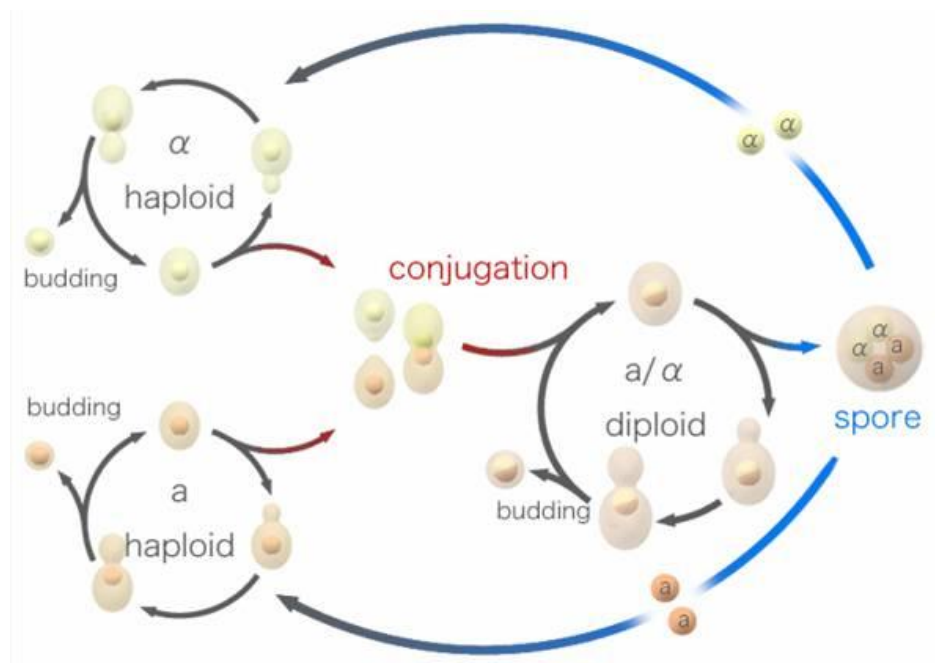
## Fungal Infections

Fungi are prevalent in the environment, many of which are pathogens that can infect plants and animals, including humans. For example, fungi are the most common cause of plant disease (Prescott *et al.*, 2005). Fungi such as *Sclerotinia sclerotiorum* can cause infection of many crops (Huang *et al.*, 2000). About fifty fungal species have been found to cause disease in humans (Prescott *et al.*, 2005), among which *Candida albicans* is the most common human fungal pathogen (Heitman and Howlett, 2008). Statistics have also shown that more than half of AIDS patients have infections caused by pulmonary *Pneumocystis carinii* (Yang and Kerdel, 2006) and the occurrence of invasive fungal infections in organ transplant patients continues to be high with a high mortality rate (Gabardi *et al.*, 2007). The rise of fungal diseases spurs many scientists on to the development of novel antifungal drugs. However, the task has not been easy due to the fact that fungal pathogens are also eukaryotic with cellular machinery very similar to that of their hosts'. This makes drug target identification difficult thereby slowing the process of drug development (Heitman, 2005).

## *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* (*S. cerevisiae*) is a unicellular fungus, commonly known as baker's yeast (Daum, 2000). As a widely used eukaryotic genetic model, it was chosen as the pathogen in this project. *S. cerevisiae* was used instead of a human pathogen, such as *Candida albicans*, because *S. cerevisiae* is the best studied eukaryotic cell (Prescott *et al.*, 2005), with its genome fully sequenced and its biology well

understood (Forsburg, 1999). Compared with *S. cerevisiae*, the genomic sequence determination of human fungal pathogen genomes is proceeding very slowly, one reason being that the genome of *S. cerevisiae* is very similar to that of human fungal pathogens, so it is unnecessary to find the genome of the human fungal pathogens (Goffeau *et al.*, 1996). *S. cerevisiae* has 16 chromosomes and a small genome size of about 12 Mbp. About 6000 genes are predicted to be in the genome, and the function of 80% of the predicted genes has been characterized (Miller-Fleming, 2008). Major pathways such as cell cycle, regulation and signal transduction first identified in *S. cerevisiae* are also conserved in higher eukaryotes (Cooper and Hausman, 2007). This has made yeast a very good genetic model for studying higher eukaryotes with more complex genomes. Besides all these advantages, yeast is a safe, fast, and facile organism to culture in the laboratory that makes it an attractive model system. Its fast reproduction cycle accelerates experiment completion (Angier, 1986). Another advantage of using *S. cerevisiae* is that it provides us with an available deletion library of more than 5000 genes that can be used in the screening process of the project.



**Figure 1: *S. cerevisiae* Life Cycle**

*S. cerevisiae* has a budding mode of growth. It is normally a diploid with a long G1 phase and overlapped S and M phases. When the cells have enough nutrients, they

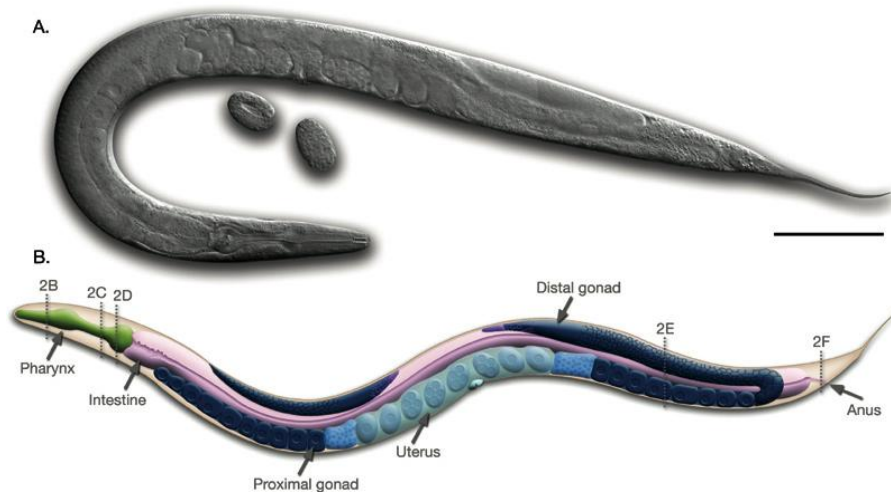


will proceed with the cell cycle. When there is a lack of nutrients, the cells will either go into a quiescent state or begin sporulation to become haploid. Haploid cells can go through conjugation to become diploid again (Pringle, 1997).

## ***Caenorhabditis elegans***

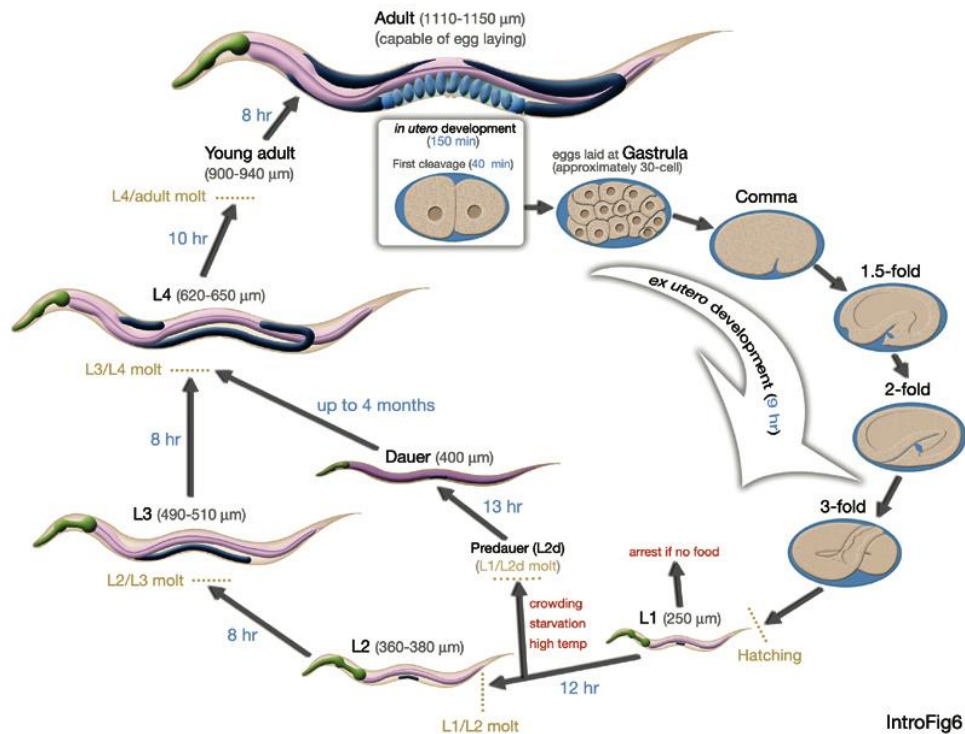
*C. elegans* are free-living in soil and are about 1mm in length. In the lab, they can be maintained on agar plates or liquid cultures. They eat bacteria in the soil and are fed with *E. coli* in the laboratory (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).

There are two sexes, hermaphrodite and male. The hermaphrodite is the most abundant sex and males compose only about 0.1% of the time (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).



**Figure 2: Anatomy of a *C. elegans* adult hermaphrodite**

The main shape is an unsegmented, cylindrical body that is tapered at the ends (shown in Figure 1). In the body wall, there is a cuticle which surrounds the worm. Within the wall there are four different systems; epithelial, nervous, muscle and excretory. The internal organs are incorporated into two systems; alimentary and reproductive. The alimentary includes the pharynx, intestine, rectum and anus. The Reproductive System consists of the somatic gonad, the germ line and the egg-laying apparatus (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).



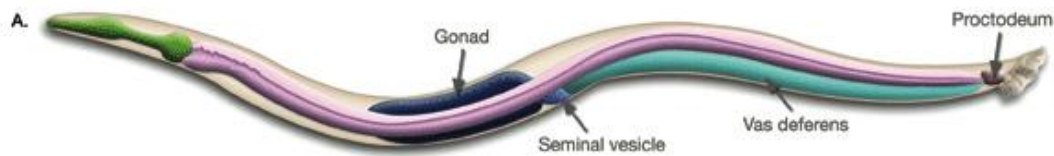
**Figure 3: Life cycle of *C. elegans***

Figure 3 shows the life cycle of a *C. elegans* hermaphrodite. This entire cycle takes only three days at 20 degrees C. The stages of the life cycle are the embryonic stage, larvae (L1-L4) stages, and adult (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).

The embryonic stage can be separated into two parts; proliferation and organogenesis/morphogenesis. During the four postembryonic larval stages, the development of the nervous system and the reproductive system occurs. If the egg hatches and there is no food available, the larva stays at the L1 stage. During L4 stage, cells for the vulva start to generate, followed by tissue morphogenesis. A transparent region can be seen in L4 larva's body where the vulva is to be formed. The adult is the fully developed reproductive form (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).

The dauer larva is formed under conditions of food limitation, starvation, or a high temperature. This stage is non-aging if the environment stays unfavorable for growth. When the dauer larva is exposed to food or a more favorable environment, then

the worm exits the dauer stage and develops to the L4 and then into an adult (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).



**Figure 4: *C. elegans* male**

Figure 4 shows anatomical structure of male *C.elegans*. Male *C.elegans* differ from hermaphrodites in a way that only one X chromosome is present in male, while two are present in hermaphrodite. The posterior half of a male *C.elegans* body is characterized by arrow-shaped tail, a structure used during mating with hermaphrodite (<http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>).

## **Model Host**

*C. elegans* has been used to provide important insights into how animals perceived threats in the environment and defend themselves against infection. This nematode has been very useful in many different aspects in biology such as developmental biology, neurobiology, and ageing (Gravato-Nobre and Hodgkin, 2005).

*C. elegans* is an ideal model host for many reasons. From the practical standpoint the advantages are low cost, easy maintenance, minimal lab space, and suitability for automated animal sorting (Gravato-Nobre and Hodgkin, 2005).

They also have a small genome size. This has helped scientists to determine the complete DNA sequence of the genome. Self-fertilization is an important aspect of *C. elegans* for genetic analysis. Another aspect that is important for studying development is that their body is transparent allowing observation of all cells and cell divisions from egg to adult. This also makes it easier to observe the infection process (Gravato-Nobre and Hodgkin, 2005).

Either in the soil or in the laboratory, *C. elegans* eats bacteria, which constitutes a convenient route for infection by microbes, as well as the application of RNA interference-based gene silencing (Gravato-Nobre and Hodgkin, 2005).

*C. elegans* is a very important model in studying innate immunity. Several pathways are involved in the nematode's innate immunity, such as DBL-1 and MAP kinase pathways ([http://www.wormbook.org/chapters/www\\_signalingimmuneresponse/signalingimmuneresponse.html](http://www.wormbook.org/chapters/www_signalingimmuneresponse/signalingimmuneresponse.html)) with a common theme in which protein cascades transduce signals in response to various extracellular signals (Mizuno *et al.*, 2004). Three MAP kinase pathways, p38 pathway, JNK pathway, and ERK pathway, are shown to be important for growth control and resistance to cellular stresses. Each cascade contains the following three classes of protein kinase: extracellular signals activate MAPKKK to phosphorylate the MAPKK, which can activate MAPK in turn (Koga *et al.*, 2000). JNK-1 (MAPK) and JKK-1 (MAPKK) have been identified in the JNK pathway, a pathway involved in several biological functions in mammalian cells (Mizuno *et al.*, 2004). All three of these MAP kinase pathways have been shown to be required for resistance of *C. elegans* to bacterial infections ([http://www.wormbook.org/chapters/www\\_signalingimmuneresponse/signalingimmuneresponse.html](http://www.wormbook.org/chapters/www_signalingimmuneresponse/signalingimmuneresponse.html); Kim *et al.*, 2004).

### ***Previous Projects in the Politz and Prusty Rao laboratories***

Previous studies have identified *S. cerevisiae* mutants that are virulent as well as worm mutants that show altered susceptibility to yeast infection. It was found that *S. cerevisiae* could infect *C. elegans* and induce the Dar phenotype. *C. elegans* produces reactive oxygen species (ROS) which are produced by the product of the *bli-3* gene, a NADPH-dependent oxidase required for normal cuticle formation. Preliminary evidence suggests that production of ROS by BLI-3 may protect *C. elegans* against yeast pathogenesis (Charu Jain and R. Prusty Rao, unpublished). *S. cerevisiae* may then use gene products of YAP 1 and SOD 1 to neutralize the effect of ROS. YAP 1 is a transcription factor in *S. cerevisiae* that is critical for oxidative-stress response (Maeta *et al.*, 2004). *SOD1* encodes the protein superoxide dismutase, which can neutralize ROS by converting superoxide radicals to hydrogen peroxide and molecular oxygen (Cox *et al.*, 2002).

These results suggest that a high-throughput assay would allow us to conduct an unbiased whole genome screen for novel fungal virulence factors as well as host immunity modulators. However, experiments on agar plates are labor intensive and time consuming. In order to screen the deletion libraries of *S. cerevisiae*, a more efficient assay is needed. We decided to try to develop a liquid culture assay in microtiter plates.

## Methodology

### **Stock Plate Maintenance**

The stocks of *C. elegans* strains were kept on 60 mm agar plates of NGM medium with an *E. coli* OP50 lawn at 16°C. Three hermaphrodites were transferred every 5 to 7 days to fresh plates. The plates were transferred to 20°C four days before egg preparations were done (Kelly and O'Brien, 2007).

### **Mutant *C.elegans***

*Fer-1(b232)* *I* mutant *C. elegans* is temperature-sensitive fertilization defective. At 20 °C, *fer-1(b232)* hermaphrodites are infertile. The *fer-1* gene product is required for  $\text{Ca}^{2+}$ -mediated membrane fusion during *C.elegans* spermiogenesis. *Fer-1* mutation alters the sensitivity to  $\text{Ca}^{2+}$ , which affects the membranous organelle fusion and results in abnormal sperm (Washington and Ward, 2006).

*Kgb-1* encodes a JNK-like MAPK in a JNK-like MAPK pathway. This JNK-like MAPK pathway is involved in the stress responses to heavy metals (Mizuno et al 2004). The *kgb-1(km21)* *IV* mutant is hypersensitive to copper ( $\text{Cu}^{+2}$ ) and cadmium ( $\text{Cd}^{2+}$ ) heavy metal ions. Most of the *kgb-1* mutants fail to grow to adult stage on plates containing 75  $\mu\text{M}$   $\text{Cu}^{2+}$  or more in four days, while the wild type *C.elegans* grow very well under these conditions (Mizuno et al., 2004).

### ***C. elegans* Egg Preparation**

Egg preparations were used to start developmentally synchronous liquid cultures in microtiter wells. Use of egg preparations also helped avoid contamination due to the use of bleach treatment to lyse worms, which sterilized the egg preparation. To make sure the worms were exposed to yeast are at the same stage of development, eggs were harvested as follows.

The egg preparation stock plates were prepared three to five days earlier to obtain the maximum number of eggs on plates. Plates were not used if they did not contain enough eggs or if the plates had starved. These plates were washed with M9 buffer to

remove eggs and worms. Then the liquid was transferred to a 15mL conical tube. It was centrifuged into a pellet for two minutes at 900xg. The supernatant was removed using a Pasteur pipette. Twelve milliliters of bleach solution, which consisted of 0.25M NaOH dissolved in a 1:4 dilution of commercial bleach in dH<sub>2</sub>O, was added to the conical tube to lyse the worms. The eggs were more resistant to the bleach solution, so they stayed intact. The tube was rocked for three minutes and centrifuged for another two minutes at 900xg. The supernatant was removed quickly, and then sterile M9 was added to wash the eggs free of traces of bleach. Between washes, samples were centrifuged for two minutes at 900xg and the supernatants were removed.

After the second wash, the pellet was resuspended in 5 mL of M9 buffer. Three 10µL drops were added to an unused agar plate to determine the average egg count for a given volume. A volume containing about fifteen to twenty eggs was then transferred into the microtiter wells and incubated at 20°C to mature.

### ***Preparation of Liquid Assay***

A single colony from a streaked plate of *E. coli* OP50 was added to 5 mL of LB and incubated in 37°C for three days. A single colony from a streaked plate of *S. cerevisiae*, such as RPY101 strain, was added to 5 mL of YPD and incubated at 30°C for three days.

After the three days, the tubes were removed from the incubators and the absorbance at 600 nm was taken. For *S. cerevisiae*, YPD was used as a blank and for *E. coli*, LB was used as a blank. The dilution for the absorbance was 1:10. It is important to take into account that *S. cerevisiae* yeast cells are very large compared to *E. coli* cells. To determine the number of cells per milliliter for *S. cerevisiae* and *E. coli*, the absorbance that was determined was converted to cells/mL using the factor  $8 \times 10^8$  cells/OD unit for *E. coli* and  $2 \times 10^7$  cells/OD unit for *S. cerevisiae*, also taking into account the dilution factor, which was 10.

The mass ratio was determined by dividing the calculated value of *S. cerevisiae*'s cells/mL by the calculated value of *E. coli*'s cells/mL, then multiply the result by 10 since one *S. cerevisiae* cell weighs 10 times more than an *E. coli* cell.

The total volume of liquid in each well was 750  $\mu$ L. The volume of *S. media* added equals to the total volume minus the volume of *S. cerevisiae* and *E. coli* together. To each well there was an addition of 1  $\mu$ L streptomycin 50 mg/mL to stop the growth of *E. coli* and bacterial contaminants. Components were added to each sample in the amounts indicated in Table 1.

Component	Added Amount
<i>E. coli</i>	50 $\mu$ L
Yeast	15 $\mu$ L - 150 $\mu$ L (depending on absorbance and concentration)
<i>S. medium</i>	750 $\mu$ L – (yeast + <i>E. coli</i> )
Streptomycin	1 $\mu$ L of 50 mg/mL
Eggs	5-10 $\mu$ L (depending on the egg count)
Total Volume	750 $\mu$ L+ egg volume + Strep volume

Table 1: Assay Set-Up

## Assay Procedure

The microtiter plates were incubated at 20°C for ten to twelve days, during which observations and data were recorded daily. The total number of live and dead worms was recorded. Worms, that were not moving or that had larvae hatched inside of bodies, were considered to be dead.

## Data Analysis using Sigma Stat

A computer program called Sigma Stat was used to plot survival percentage as a function of time. The statistics that were used is called LogRank. This compares the survival distributions of two samples. It constructed by comparing the observed and expected number of events in one of the groups at each observed event time and then adding these to obtain an overall summary across all time points where there is an event.

Data were arranged in three columns; group, time and status. The group was each condition to be compared. Time was the number of days since plating of eggs. Status was alive or dead. For each day, each death was recorded in a separate line in the spreadsheet. Each worm had to be entered in as an individual. For every worm that had died that day there would be a one for the status and the number of rows for that day would coincide with the number of worms that died that day. For each row there would



be the group name, the day, and a one for the status. If no worms died within a 24 hour period then there would be a group name, the day, and for the status it would be left blank.

To plot the graph and calculate significance between groups, the log-rank test was used. Groups to be compared pairwise were chosen, and because worms could not escape the microtiter wells, no data were censored (the set-up is also shown in Appendix 2).

## **$\text{Cu}^{2+}$ plate preparation**

Agar plates of NGM medium with 75  $\mu\text{M}$   $\text{Cu}^{2+}$  were prepared by adding cupric sulfate ( $\text{CuSO}_4$ ) into standard NGM medium before pore the plates. The amount of cupric sulfate added was calculated using the formula below:

Weight of cupric sulfate =

$$75 \mu\text{M} \times \text{Volume of the solution} \times \text{molecular weight of cupric sulfate}$$

The calculated amount of cupric sulfate was dissolved in  $\text{dH}_2\text{O}$  first. The volume of  $\text{dH}_2\text{O}$  used was then deducted from the NGM medium recipe. For example, if 75 mL  $\text{dH}_2\text{O}$  was used to dissolve cupric sulfate, 75 mL less of  $\text{dH}_2\text{O}$  was added when mixing NGM medium. The cupric sulfate solution was added into NGM medium after being autoclaved.

## ***N2 male C. elegans***

*N2* males were obtained by leaving L4 stage larvae at 27.5°C overnight. The elevated temperature could increase the occurrence of males in the population. Ten L4 were placed on a new plate. The plate was moved back to 20°C on the second day. The progeny of these ten worms were observed and selected for males. A male stock plate were started with putting ten *N2* hermaphrodites (L4 stage or younger) and five *N2* males, matching the age of hermaphrodites, at 16°C. This procedure was repeated daily until a good stock of *N2* males was obtained.

## ***Fer-1; kgb-1* double mutant construction**

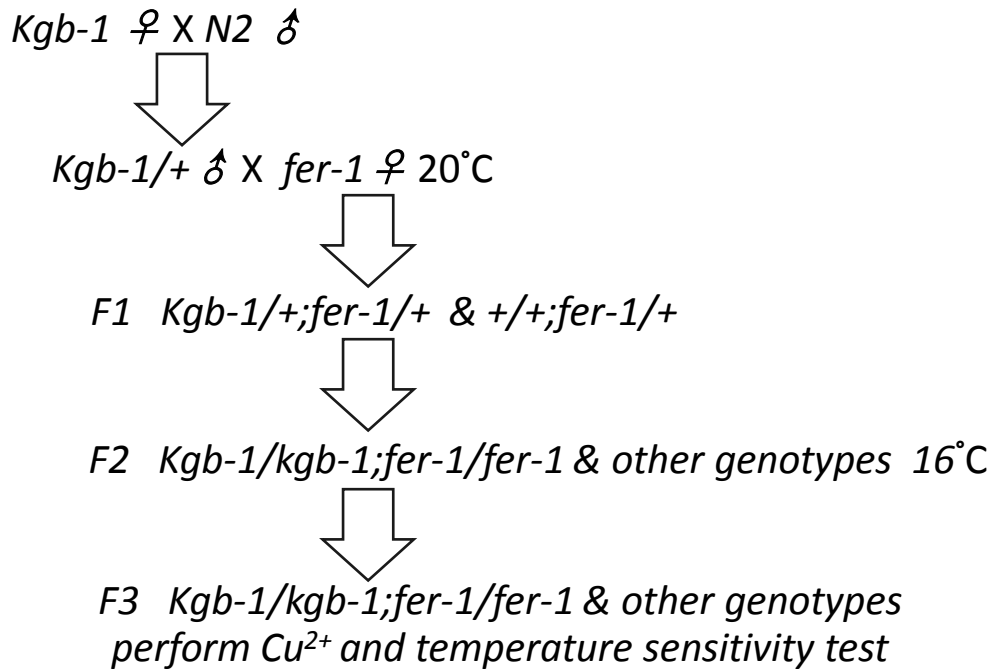
Since *kgb-1* males did not mate well with any hermaphrodites in our preliminary tests, ten *kgb-1* hermaphrodites (L4 stage or younger) and five *N2* males were put on one

plate for crossing first. Five of the resulted male progeny, heterozygous for *kgb-1*, were then crossed with ten *fer-1(b232)* hermaphrodites (L4 stage or younger) at 20°C on another plate. In this way, the offspring on this plate could only be from crossing, because the *fer-1* hermaphrodites are infertile at 20°C. Ten of the F1 hermaphrodite offspring were picked onto plates, one hermaphrodite per plate. The F1 hermaphrodites were picked unbiased for *kgb-1*. The plates were kept at 20°C to ensure that they were not offspring from self-fertilized *fer-1* hermaphrodites, and were moved to 16°C as soon as fertilized eggs (F2) were found on plates. F2 worms were allowed to grow to L4 stage and were then singled onto new plates. Approximately sixty F2 hermaphrodites were picked, six from each F1 plate. The F3 eggs of each F2 were used in Cu<sup>2+</sup> and temperature sensitivity tests. F2 plates were marked with numbers for future reference and maintained at 16°C.

In the Cu<sup>2+</sup> sensitivity test, for each F2, about twenty eggs were picked onto a plate containing 75 µM Cu<sup>2+</sup>, labeled with the number on the F2 plate. The plates were left at 16°C for growth and observed after a week. If all worms on a single plate failed to reach the adult stage, the F2 that laid these eggs was determined to be homozygous *kgb-1*.

After *kgb-1* homozygous F2 were found, eggs of F3 left on the same F2 plate were picked again for the temperature sensitivity test. Again, for each *kgb-1* homozygous F2, about twenty eggs were picked onto a standard NGM plate, labeled with the number on the F2 plate. The plates were left at 20°C for growth and observed after about five days. If all the eggs laid by F3s were infertile, then the F2 was determined to be *fer-1* homozygous. Plates with both fertile and infertile eggs were determined to be heterozygous for *fer-1*. The temperature sensitivity test could be carried out again to find additional homozygous *fer-1*, by singling offspring from the F2 plates accordingly and testing the eggs of these plates. After performing Cu<sup>2+</sup> and temperature sensitivity tests, the *kgb-1*; *fer-1* double mutant could be identified.

A simplified diagram of construction procedure is shown below.



**Figure 5: Double mutant construction strategy**

### **Freezing *kgb-1*; *fer-1* double mutant**

Freezing of *kgb-1*; *fer-1* double mutant was performed as follows. Larvae of *kgb-1*; *fer-1* double mutant were washed off from a recently starved plate, with a majority of larvae being L1 or L2. 2.2 ml M9 buffer (Brenner, 1974) was used and the suspension was placed in a sterile conical tube with cap. Equal volumes of sterile 2X S medium ([http://www.med.yale.edu/mbb/koelle/protocols/protocol\\_liquid\\_culture.html](http://www.med.yale.edu/mbb/koelle/protocols/protocol_liquid_culture.html)) and sterile 30% glycerol (w:v) were mixed to obtain 1X S medium containing 15% glycerol (w:v). 1.6 ml of larval suspension and 1.6 of the S. medium-glycerol solution were drawn into a new conical tube for mixing. This mixture was then separated equally into six freezer vials, which later were placed at -80 °C overnight. One vial was taken out and thawed the next day to determine viability of the frozen stock. After a stock plate was successfully started from this vial, the rest of the vials were moved into liquid nitrogen for storage.

## Results

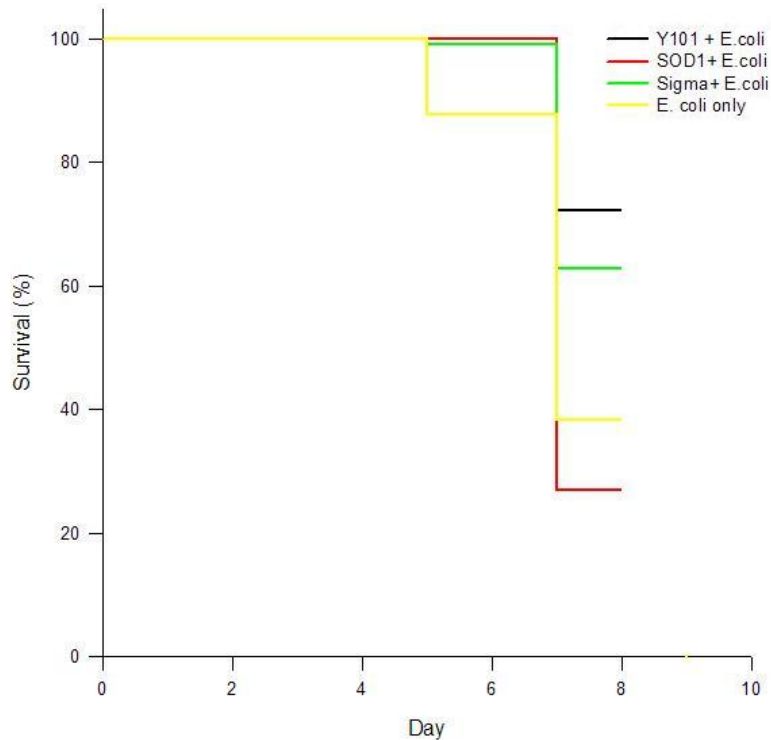
All the Figures in this section are survival curves plotted using Sigma Stat.

### ***C.elegans arrested at L1 stage when S. cerevisiae served as the only food***

In wells that *C.elegans* were fed with 100% *S. cerevisiae*, the eggs hatched but were all arrested at L1 stage in development (data not shown). This is consistent with results from previous projects on agar plates.

## ***S. cerevisiae* Mutants**

Wild type *S. cerevisiae* (Y101), *S. cerevisiae* SOD1 mutant and Sigma strain (Y196) were used in this experiment. *Sod1Δ* is a superoxide dismutase deletion mutant. Sigma (Y196) is a wild strain that is more virulent than Y101. The mass ratio of all *S. cerevisiae* to *E.coli* was 1:30. NGM was used in all the wells.



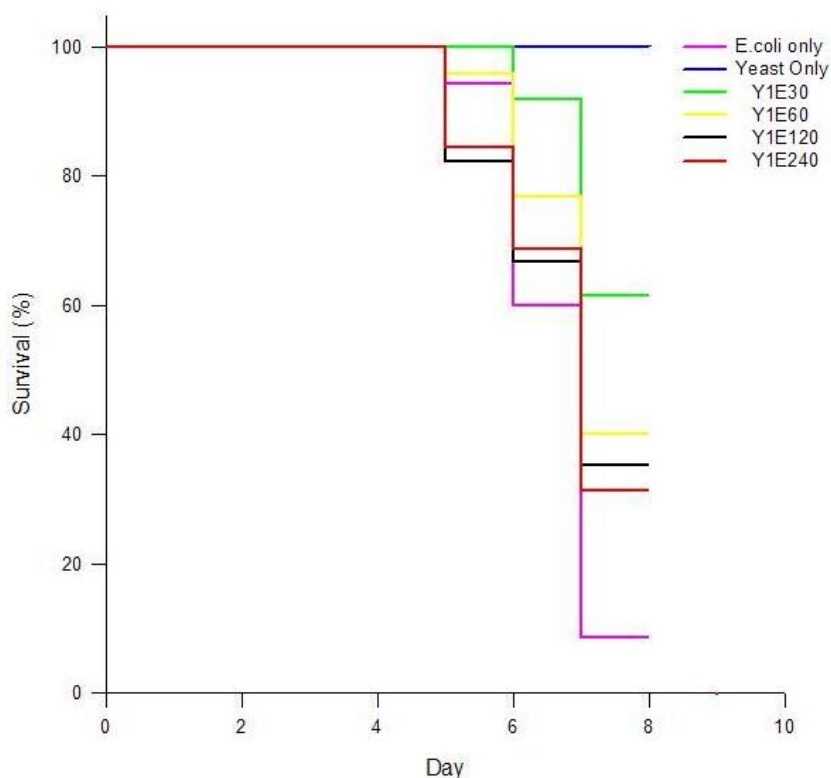
**Figure 6: Survival curve of *C. elegans* with *S. cerevisiae* mutants and *E.coli* in NGM**

The Y axis is the percentage of live *C.elegans* each day. The X axis is the time of experiment in days. The *C. elegans* were counted every day for 8 days. The mass ratio of *S. cerevisiae*: *E.coli* was 1:30.

Results of this experiment are shown in Figure 6. *C. elegans* grown on Y101 and SOD1 were significantly different ( $P < 0.001$ ). Sigma and Sod 1Δ were significantly different from each other ( $P < 0.001$ ). Sigma was not significantly different from the Y101 control ( $P = 0.141$ ). However, *C. elegans* grown on Sigma, which were expected to die faster than Sod 1Δ, actually had a higher survival percentage by the end of the experiment. This was probably due to difficulty encountered in counting. *S. cerevisiae* became very dense in the center of wells during experiments, which made the field under the microscope unclear for counting for all the conditions.

## Varying Mass Ratios

The mass ratios used for the first experiment were 1:30, 1:60, 1:120, and 1:240 *S. cerevisiae*: *E. coli*. After this first experiment the media was changed because the *S. cerevisiae* was very thick due to the use of rich medium NGM. *S.* medium was used subsequently because it contains no carbon or nitrogen source for *S. cerevisiae* to grow and the counting would be more accurate.



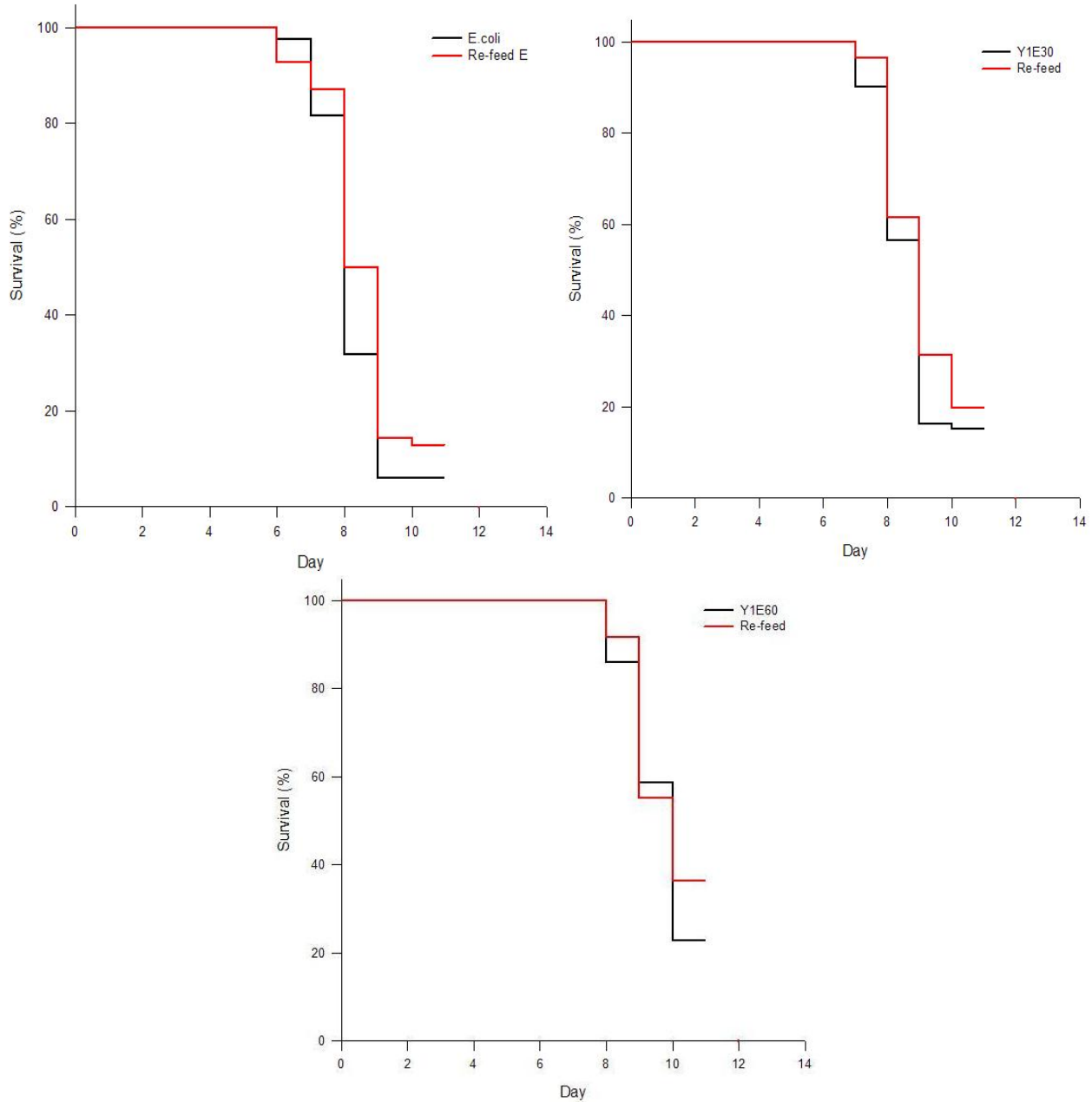
**Figure 7: Survival curve of *C. elegans* with different mass ratio of *S. cerevisiae* to *E. coli* in *S. medium***

Y1E30 is a 1:30 mass ratio of *S. cerevisiae* to *E. coli*. The same notation was used for the other ratios of 1:60, 1:120, and 1:240. The Y axis is the percentage of live *C. elegans* each day. The X axis is the time of experiment in days. The *C. elegans* were counted every day for 8 days.

Results of an experiment to test the effects of different mass ratios of *S. cerevisiae* and *E. coli* are shown in Figure 7. With a better view of the plate, 1:60, 1:120 and 1:240 mass ratios of *S. cerevisiae* to *E. coli* were significantly different from 1:30 ( $P = <0.001$ ), using pair wise comparisons. However, the majority of dead *C. elegans* had larva hatched

within their bodies. Whether the *C.elegans* died from *S. cerevisiae* infection or from the hatching larva was unclear.

For the next experiment (Figure 8), *C.elegans* were fed on the third day of the experiment with the same amount of *S. cerevisiae* used to start the experiment. Control wells with *E.coli* only were fed with same amount *E.coli*.



**Figure 8: Survival curves of *N2 C.elegans* at different mass ratios of *S. cerevisiae* to *E. coli* with re-feeding**

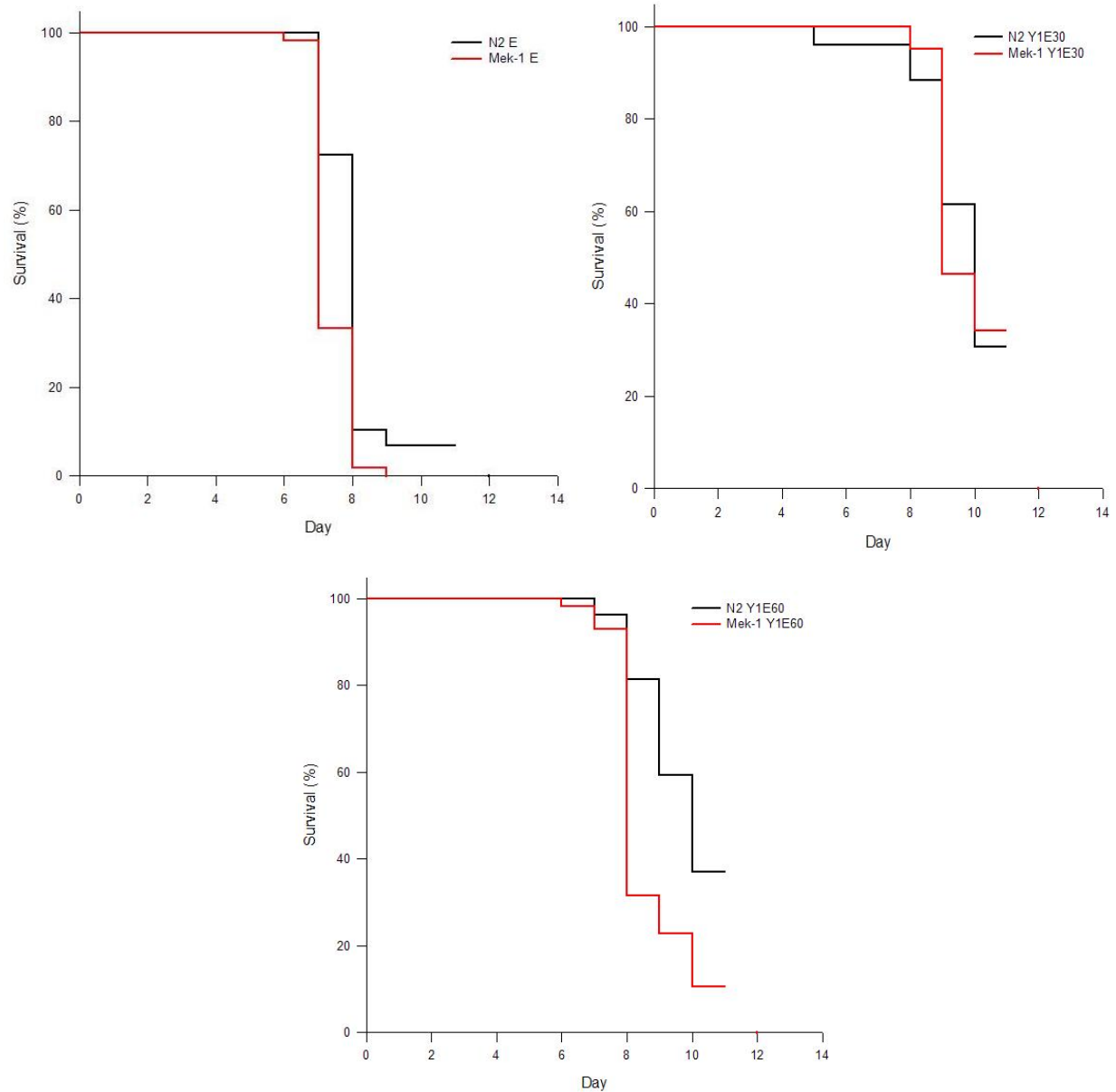
Y1E30 is the mass ratio of *S. cerevisiae* to *E.coli*, 1:30. The same notation is used for 1:60 mass ratios. The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment in days



*C.elegans* in re-feeding wells did live longer than the non-feeding ones in the *E.coli* only condition ( $P = 0.038$ ). But in 1:30 and 1:60 mass ratios, there was no significant differences between re-feeding and non-feeding conditions (for 1:30,  $P = 0.176$ ; for 1:60,  $P=0.134$ ). However, dead *C.elegans* with larva hatched within bodies were still observed during the experiment.

### ***C. elegans* Mutants**

The *C. elegans* mutants that were used were *mek-1(ks54)* and *fer-1(b232)*. *Mek-1* encodes a MAP kinase that is required for resistance to killing by *S. cerevisiae* in agar plate cultures (Meijiang Yun, unpublished results).



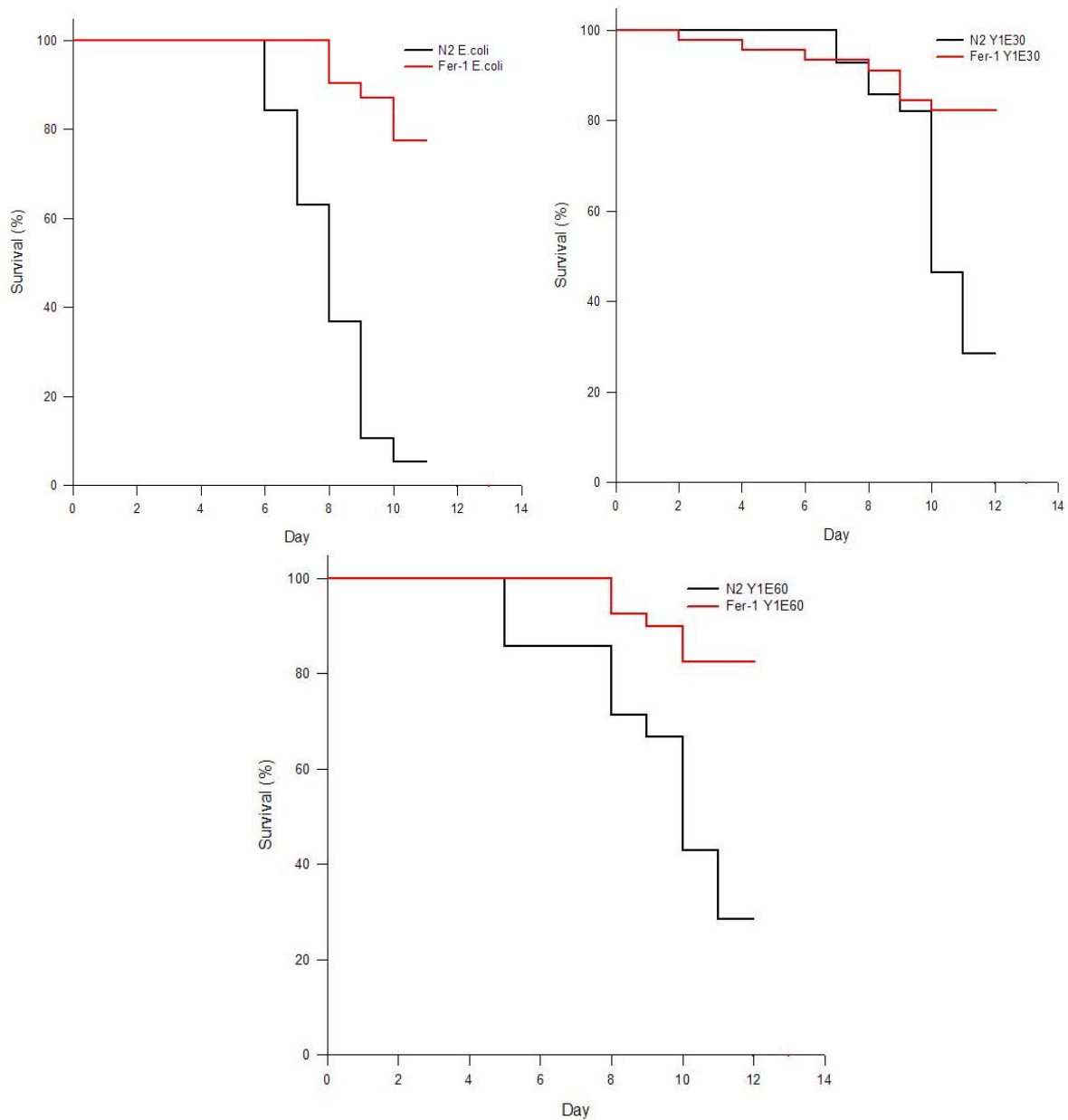
**Figure 9: Survival curves of N2 and *mek-1* *C.elegans* at different mass ratios of *S. cerevisiae* to *E.coli* with re-feeding**

The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment. The number of worms was counted each day for 11 days.

The experiment was done with three different conditions: an amount of equivalent to that mixed with yeast in other samples, a mass ratio of *S. cerevisiae* to *E. coli* of 1:30, and a mass ratio of *S. cerevisiae* to *E. coli* of 1:60. Results are shown in Figure 8. *Mek-1* died significantly faster than *N2* ( $P = <0.001$ ) in both *E.coli* control and 1:60 mass ratio conditions. The P value of 1:30 mass ratio was 0.958, from which we concluded that the

1:30 survival curve is not great enough to exclude the possibility that *mek-1* lived longer than the N2 strain is due to random sampling variability. Both *mek-1* and N2 had many dead *C.elegans* with larva hatched inside the bodies.

*Fer-1* is a *C. elegans* mutant that is temperature-sensitive fertilization defective. At 20 °C, *fer-1(b232)* hermaphrodites are infertile.



**Figure 10: Survival curves of N2 and *fer-1* *C.elegans* at different mass ratios of *S. cerevisiae* to *E.coli* at 20 °C with re-feeding**

The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment in days. The *C. elegans* were counted everyday for 12 days.

The experiment was done with three different conditions: same amount of *E. coli*, a mass ratio of *S. cerevisiae* to *E. coli* of 1:30, and a mass ratio of *S. cerevisiae* to *E. coli* of 1:60. Y1E30 notation means a *S. cerevisiae* to *E. coli* mass ratio of 1:30. The same notation is used for mass ratio of 1:60. *Fer-1* lived significantly longer than N2 in the

experiment (All curves have a P value of  $<0.001$ ). About 80% of the *fer-1 C.elegans* lived till the 13<sup>th</sup> day of the experiment.

### ***Kgb-1; fer-1 C. elegans double mutant was constructed successfully***

After performing the construction strategy described in Methodology, three clones of *kgb-1; fer-1* double mutant were found. As expected for a *kgb-1* mutant, eggs of the putative double mutant strain failed to reach adult stage on plates containing 75  $\mu\text{M}$   $\text{Cu}^{2+}$ . As expected for a *fer-1* mutant, when grown at 20°C, their offspring did not lay fertilized eggs. These results verified that the three clones are true *kgb-1; fer-1* double mutants.

## Discussion

Using a liquid culture assay would be ideal for identifying fungal virulent factors because it would make the high-throughput screening of the deletion library more efficient. However, the assay has not been fully developed.

When using different mass ratios of *S. cerevisiae* to *E. coli*, *C. elegans* did not die significantly faster at a lower concentration of *S. cerevisiae* compared to a higher concentration of *S. cerevisiae*. Death could be accounted for fertilized eggs hatching inside the body. While viewing this, there was uncertainty of whether the *C. elegans* died because of *S. cerevisiae* or because of starvation. When the environment is not ideal, *C. elegans* does not lay eggs (Schafer, 2005).

When observing the results obtained with *N2* and *mek-1*, *mek-1* died faster compared to *N2*. There were other factors that influenced the dying of the *C. elegans* such as larva hatching inside of the body. This mutation in the MAP kinase cascade in the immune system may still be useful for high throughput assays because it is weaker and more susceptible to infection.

To eliminate death due to fertilized eggs hatching within the worm's body, we used *fer-1*, a temperature-sensitive fertilization-defective mutant. At the restrictive, 20°C, *fer-1(b232)* sperm are inactivated and eggs consequently are not fertilized. This would prevent the effects of *S. cerevisiae* infection from being obscured and allow the real effects of yeast to be observed. The *fer-1 C. elegans* stayed alive throughout the experiment; only about 20% of the *fer-1 C. elegans* died within 12 days while more than 70% of the *N2* died. The only reason that 100% of the *N2* did not die is that the experiment was terminated at 12 days. This experiment allowed us to conclude that wild type *C. elegans* was not dying due to *S. cerevisiae* but due to the fertilized eggs hatching within the body.

From the results of experiments using *fer-1 C. elegans* mutants, a *kgb-1; fer-1* double mutant of *C. elegans* would be a good strain in which to study fungal infections in worms. *Fer-1(b232)* mutant *C. elegans* do not lay any fertilized eggs at 20°C, the temperature used for previous experiments, which helps excluding the death-causing factor of fertilized eggs hatching inside *C. elegans* bodies from experimental results. *Kgb-1* mutant *C. elegans* was shown by Meijiang Yun (unpublished results) to show

enhanced susceptibility to fungal infection; a *kgb-1* mutant was previously shown to be important for innate immune responses to bacterial infections (Kim *et al.*, 2004). Therefore if *kgb-1; fer-1* also shows enhanced susceptibility to yeast infection, it is likely to be because of a defect in innate immunity.

## Future Experiments

With the newly available *kbg-1; fer-1* *C. elegans* double mutant, observational analysis on intestinal lipofuscin auto-fluorescence intensity, using BY4742 yeast strain expressing RFP, can be done to visualize the infection caused by *S. cerevisiae*. In the absence of matricide from internal hatching, more significant features of yeast pathogenesis might be revealed. Survival experiments on agar plates can also be done to see if this double mutant is as susceptible as a *kbg-1* single mutant. By comparing the results from these experiments, a better view of intestinal infection in *C. elegans* can be obtained, and the real death-causing factor, either matricide or fungal infection, can be determined. Therefore, the effect of *S. cerevisiae* on *C. elegans* is expected to be represented more accurately in future experiments.



## Appendix 1: Raw Data

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
E.coli only	65	86	105	106	98	64	15
Yeast:E.coli 1:30	48	113	112	104	108	100	21
Yeast:E.coli 1:60	68	147	142	141	135	107	48
Yeast:E.coli 1:120	136	151	154	153	125	103	24
Yeast:E.coli 1:240	80	100	95	98	82	67	6

Various Mass Ratios Without Feeding

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
E.coli only	61	81	82	82	82	80	67	26	5	5
E. coli only Fed	60	68	69	69	69	64	60	34	9	8
Yeast:E.coli 1/30	53	87	92	92	92	92	83	52	15	14
Yeast:E.coli 1/30 fed	54	81	86	86	86	86	83	53	27	17
Yeast:E.coli 1/60	86	109	111	114	114	114	114	98	67	26
Yeast:E.coli 1/60 fed	63	96	96	96	96	96	96	88	53	35

Various Mass Ratios With Feeding

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Y101 + E.coli	48	67	101	106	91	68
SOD1+ E.coli	77	99	146	168	192	52
Sigma+ E.coli	39	97	129	116	115	73
E. coli only	37	102	107	128	114	50

Different Yeast Mutants and Stains

N2	day 1	day2	day 3	day4	day5	day6	day7	day8	day9	day10
E.coli re-feed	24	28	28	28	29	29	21	3	2	2
1Y:30E re-feed	19	25	26	26	25	25	25	23	16	8
1Y:60E re-feed	18	25	27	28	28	28	27	23	16	10
Mek-1	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day10
E.coli re-feed	38	49	58	57	57	56	19	1	0	0
1Y:30E re-feed	13	27	35	41	41	41	41	34	19	14
1Y:60E re-feed	28	40	54	54	57	56	53	18	13	6

*mek-1* and N2 With Feeding

N2	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day 10	day 11
E.coli re-feed	16	17	19	19	19	16	12	7	2	1	1
1Y:30E re-feed	19	21	28	29	29	29	27	25	23	13	8
1Y:60E re-feed	16	26	21	21	18	18	18	15	14	10	6

fer-1	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day10	day 11
E.coli re-feed	23	31	31	31	31	31	31	28	28	24	24
1Y:30E re-feed	15	32	42	42	42	42	42	41	38	37	37
1Y:60E re-feed	23	31	37	37	40	40	40	37	36	33	33

*fer-1* and N2

## Appendix 2: SigmaStat Set-Up

SigmaStat - [Data 1\*]

File Edit View Format Tools Graph Statistics Transforms Window Help

Survival LogRank

50%

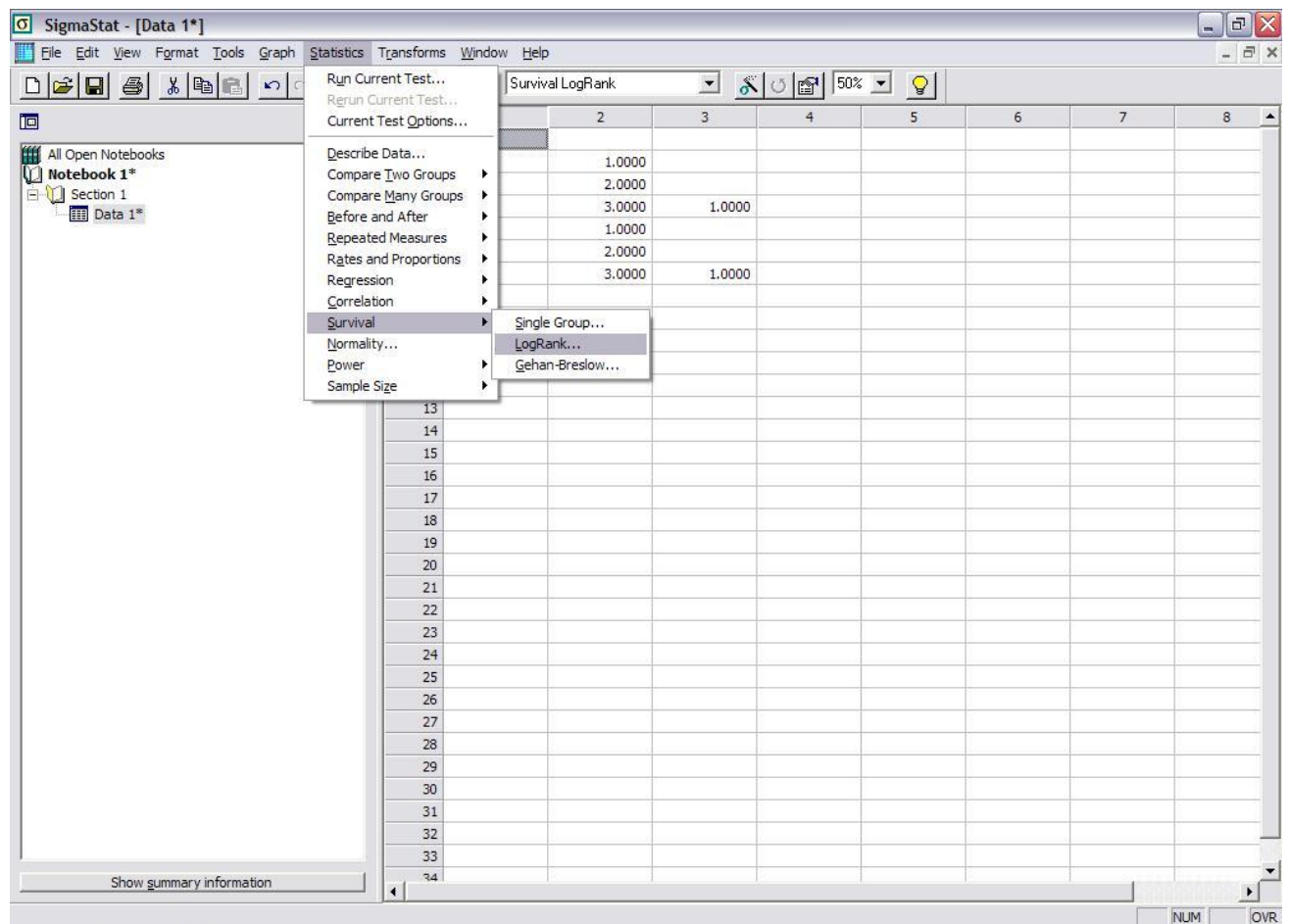
All Open Notebooks

- Notebook 1\*
  - Section 1
    - Data 1\*

	1	2	3	4	5	6	7	8
1								
2	N2	1.0000						
3	N2	2.0000						
4	N2	3.0000	1.0000					
5	mek-1	1.0000						
6	mek-1	2.0000						
7	mek-1	3.0000	1.0000					
8								
9								
10								
11								
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34								

Show summary information

Step 1: Enter Data in Columns



Step 2: Select LogRank Test

SigmaStat - [Data 1\*]

File Edit View Format Tools Graph Statistics Transforms Window Help

Survival LogRank

50%

All Open Notebooks

Notebook 1\*

Section 1

Data 1\*

	1	2	3	4	5	6	7	8
1								
2	N2	1.0000						
3	N2	2.0000						
4	N2	3.0000	1.0000					
5	mek-1	1.0000						
6	mek-1	2.0000						
7	mek-1	3.0000	1.0000					
8								
9								
10								
11								
12								
13								
14								
15								
16								
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18								
19								
20								
21								
22								
23								
24								
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26								
27								
28								
29								
30								
31								
32								
33								
34								

Pick Columns for Survival LogRank

Select the format of your data.

1-Time 1	2-Status 1	3-Time 2	4-Status 2
0.00 CENSOR	1.00 FAILURE		
1.00 FAILURE	1.00 FAILURE		
1.00 FAILURE	1.00 CENSOR		
2.00 FAILURE	2.00 FAILURE		
3.00 CENSOR	3.00 FAILURE		
5.00 FAILURE	7.00 CENSOR		
6.00 FAILURE	7.00 FAILURE		
7.00 CENSOR	10.00 CENSOR		
8.00 FAILURE	10.00 CENSOR		
8.00 CENSOR	10.00 CENSOR		

The indexed data format places multi-group survival data in three columns.

Data Format

Indexed

Help Cancel Back Next Finish

Show summary information

For Help, press F1

NUM OVR

Step 3: Select Data Format

SigmaStat - [Data 1\*]

File Edit View Format Tools Graph Statistics Transforms Window Help

Survival LogRank

50%

All Open Notebooks

Notebook 1\*

Section 1

Data 1\*

	1	2	3	4	5	6	7	8
1								
2	N2	1.0000						
3	N2	2.0000						
4	N2	3.0000	1.0000					
5	mek-1	1.0000						
6	mek-1	2.0000						
7	mek-1	3.0000	1.0000					
8								
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28								
29								
30								
31								
32								
33								
34								

1-1, me 1 2-Status: for group, time, and status.

Select data by clicking worksheet columns.

1 0.00 CENSOR

2 1.00 FAILURE

3 1.00 FAILURE

4 2.00 FAILURE

5 3.00 CENSOR

Data for Status: Column 3

Selected Columns

Group: Column 1

Time: Column 2

Status: Column 3

Help Cancel Back Next Finish

Show summary information

For Help, press F1

NUM OVR

Step 4: Select Group, Time, and Status Columns

SigmaStat - [Data 1\*]

File Edit View Format Tools Graph Statistics Transforms Window Help

Survival LogRank

50%

All Open Notebooks

Notebook 1\*

Section 1

Data 1\*

	1	2	3	4	5	6	7	8
1								
2	N2	1.0000						
3	N2	2.0000						
4	N2	3.0000	1.0000					
5	mek-1	1.0000						
6	mek-1	2.0000						
7	mek-1	3.0000	1.0000					
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33								
34								

Show summary information

For Help, press F1

NUM OVR

**Pick Columns for Survival LogRank**

Select groups for analysis.

1-Group	2-Time	3-Status
CLINIC 1	1.00	1.00
CLINIC 1	4.00	1.00
CLINIC 1	2.00	1.00
CLINIC 1	7.00	0.00
CLINIC 2	2.00	1.00
CLINIC 2	2.00	1.00
CLINIC 2	5.00	1.00
CLINIC 2	6.00	1.00
CLINIC 2	7.00	1.00

Use the drop-down list to pick at least two groups to be analyzed and compared.

Data for Group 3: **mek-1**

Selected Groups

Group 1: N2

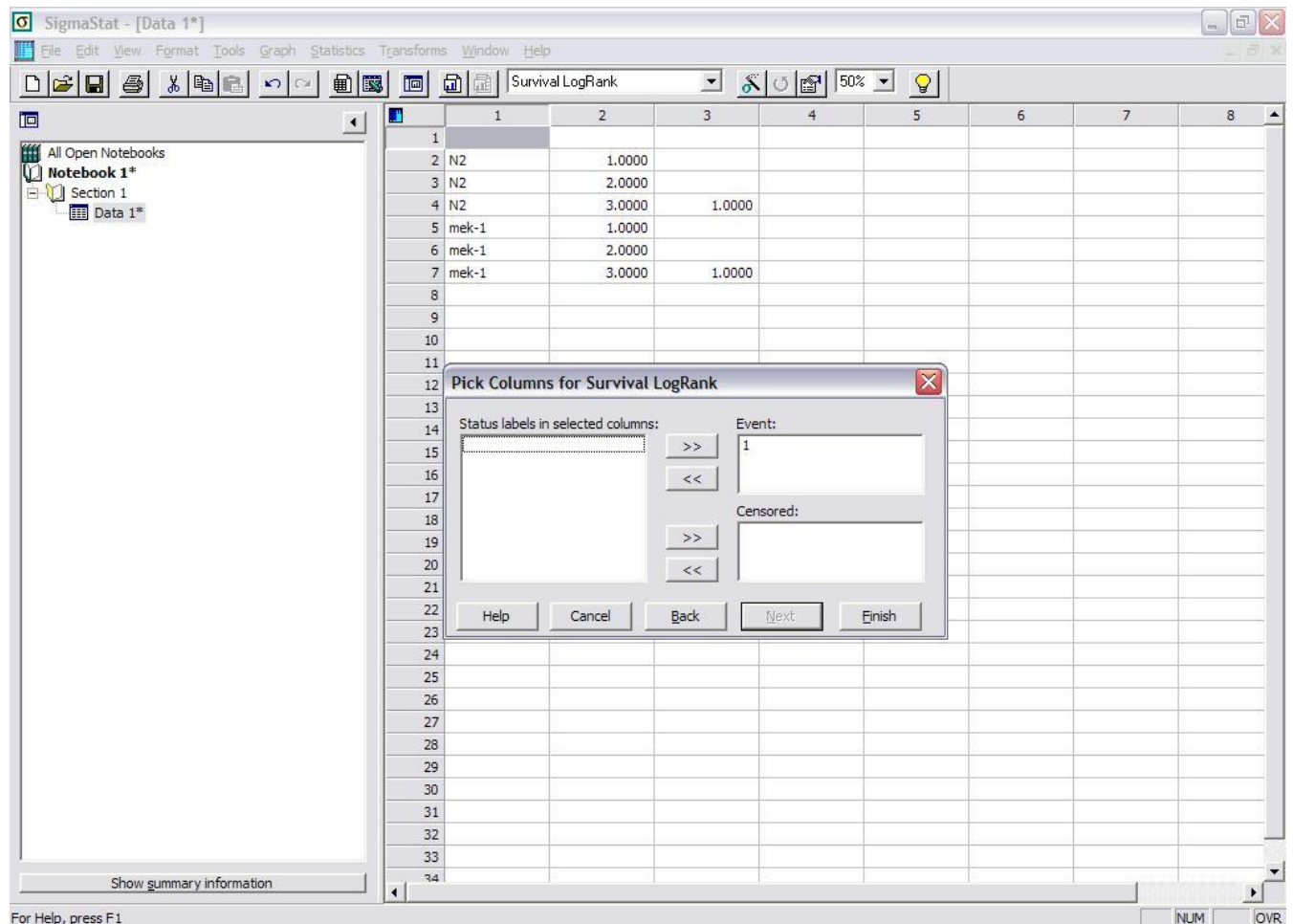
Group 2: mek-1

Group 3:

☐ Select all groups

Help Cancel Back Next Finish

Step 5: Select Groups for Comparison



Step 6: Select Event (nothing is censored) and Click Finish



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